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Osteoclasts and a Small Population of Peripheral Blood Cells Share Common Surface Antigens

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Summary. Several methods have been tried to identify mononuclear osteoclast precursors. We used a panel of 13 osteoclast-recognizing monoclonal antibodies (mabs) for the identification of osteoclast precursor cells from the bone, bone marrow, and peripheral blood of egg laying hens. Almost all mabs stained some mononuclear cells in the bone. Seven mabs recognized few mononuclear cells in the bone marrow and five mabs gave the positive immunofluorescence reaction in the white blood cell fraction. Possible immediate osteoclast precursor cells differing from osteoclasts in their densities were identified in the bone. Three mabs (K38, K52, and K70) stained the same amount of mononuclear cells (2.6–3.4%) enriched in Percoll density centrifugation. Of the monoclonal antibodies that recognized few cells in blood, K41 stained only osteoclasts. K47 and K52 also recognized some mononuclear cells in the bone marrow. Other monoclonal antibodies K51 and K70 were more unspecific, since they stained cells derived from other tissues. Blood cells detected with these different monoclonal antibodies were negative for tartrate-resistant acid phosphatase (TRAP). On the basis of our results, we suggest that there is in the blood a specific TRAP-negative cell population, which is a good candidate for osteoclast precursor.

Key words. Osteoclast — Monoclonal antibodies — Osteoclast precursor.

Mononuclear osteoclast precursor cells have been recognized in bone marrow [1–10] and spleen [8]. In these studies multinucleated osteoclast-like cells with many features of osteoclast have been generated. For example, formed multinuclear cells were able to resorb bone [8–10] and they responded to osteotropic hormones [10]. Furthermore, the studies with osteopetrotic animals have suggested that there are precursors of osteoclast in bone marrow and spleen, since these cells were able to restore permanently the capacity to resorb bone and calcified cartilage in mice with inherited osteopetrosis [11–14].

The exact origin of the osteoclast is still unknown. Mature macrophages are known not to be able to differentiate to osteoclasts [3, 4]. There is a dispute over whether osteoclast is derived from the hematopoietic stem cell, or monocyte, or monoblast or promonocyte, the two latter of which are less differentiated precursors of the monocyte [15]. It has been reported that along with the purification of the hematopoietic stem cell the osteoclast-forming capacity was increased [6]. Pluripotent hematopoietic stem cells, isolated from either thymus or bone marrow, also gave rise to osteoclasts [7]. On the other hand, the evidence that osteoclast precursor cells are present in blood was provided by the studies with chick-quail chimeras [16, 17] and with the parabiotic union of experimental animals [18]. There is also strong evidence suggesting that osteoclasts are derived from mononuclear phagocytes. This statement is based on the common antigenic structures [19–22], the similar patterns of distribution of cytoskeletal structures [23], and the similar adherence characters [23] in both of these cells. Furthermore, the facts that monocytes and macrophages are attracted to bone [24, 25] and that monocytes are able to fuse with osteoclasts [26–28] give more support for the suggestion that osteoclasts are derived from mononuclear phagocytes. Thus, it seems evident that osteoclasts derive from

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hematopoietic stem cells and are closely related to the cells of the mononuclear phagocyte lineage. To obtain more data on this problem we have developed several monoclonal antibodies against isolated chicken osteoclasts. In this paper we show data which suggest that osteoclast precursors are present in the peripheral blood of egg laying hens.

Materials and Methods

Isolation of Osteoclasts

The crude osteoclast-fraction was isolated from chickens weighing approximately 500 g each. The chickens were kept on a normal diet. Epiphysis were cut off from tibias and femurs in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) at 0–4°C. Bone slices were digested with collagenase (type IA, 750 U/ml, Sigma Chemical Co, St. Louis, MO,USA) in the above-mentioned medium for 1 hour at 37°C. After digestion bone slices were rinsed with DMEM-5% FCS using a syringe to remove cells from the endosteal surface of the bone. The cell suspension was passed through 145- and 50-μm Nybolt nylon filters (Swiss Silk Bolting Cloth Manufacturing Co., Zu-rich, Switzerland) to remove large fragments and centrifuged for 10 minutes at 120 *g*. The cell pellets were suspended in a small volume of DMEM-5% FCS and layered onto a five-step (10–50%) Percoll (Pharmacia AB, Uppsala, Sweden) density gradient [21]. Tubes were centrifuged at 200 *g* for 25 minutes at 4°C. The two upper layers consisting mainly of osteoclasts were harvested by pipette and washed once with phosphate-buffered saline (PBS). After centrifugation the cell pellets were suspended and used for immunization.

Production of Monoclonal Antibodies Against Chicken Osteoclasts

Eight-week-old female BALB/c mice were used for immunization. 150,000–300,000 isolated osteoclasts in PBS with approximately 70% purity were injected intraperitoneally 4 times during every 10 days.

Three days after the last booster injection the mouse was killed, and the spleen was removed aseptically and placed on a petri dish containing HEPES-buffered DMEM. Fusion was performed with some modifications as previously described [29]. The ratio of spleen cells/P3-NS1-Ag4-1 myeloma cells was 5:1. Cloning of hybridomas was performed using semisolid methylcellulose [30]. After the fusion hybridoma colonies were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2–3 weeks, whereafter the colonies were visible to the naked eye. The colonies were examined by inverted microscope to make sure that they were composed of cells and they were far enough from each other. Selected colonies were picked up by a pipette using a shifting mirror under the dish and transferred to 96 multiwell dishes, where cultivation was carried on until hybridomas reached 50–100% confluency.

Screening of Monoclonal Antibodies by Immunofluorescence Technique

Bone and bone marrow cells were otherwise prepared as described above but without Percoll density gradient centrifugation. The staining of the cells was performed modifying the method as described previously [31]. One-hundred-microliter cell suspensions (approximately 2–3 × 10^7 cells/ml) in DMEM-5% FCS and 200 μl culture medium or 1:50 diluted ascitic fluid were incubated for 30 minutes at 4°C. Cells were washed twice with cold DMEM-5% FCS and resuspended in 100 μl medium containing 1:50 diluted fluorescein isothiocyanate (FITC)–conjugated antimouse immunoglobulins (DAKOPATTS a/s, Glostrup, Denmark). Cells were incubated for 20 minutes at 4°C and washed once with cold DMEM-5% FCS and once with cold 3% bovine serum albumin (BSA) in PBS. Finally, the cells were suspended in 50–70 μl of 3% BSA-PBS, placed on a glass slide, covered with a coverslip, and examined under the fluorescence microscope.

Determination of Immunoglobulin Class and Subclass

Immunoglobulin class and subclass were determined by Ouchterlony immunodiffusion analysis using a Mouse Antibody Isotyping Kit for Immunodiffusion (Zymed, CA, USA).

Preparation of Bone Mononuclear Cells

Bone mononuclear cells were otherwise prepared as described in Isolation of Osteoclasts, but 40–50% Percoll layer was harvested instead of 10–20%.

Preparation of White Blood Cells from the Peripheral Blood

White blood cells were separated from the peripheral blood of egg laying hens using the Ficoll Paque-technique (Pharmacia AB, Uppsala, Sweden). Cells were first separated from serum, washed with PBS, suspended in 2 ml of DMEM-5% FCS, and layered over 8 ml Ficoll Paque. The tubes were centrifuged at 800 g for 15 minutes at 20°C. Lymphocytes were concentrated at the top of the Ficoll whereas blood red cells and granulocytes settled at the bottom of the tube. Lymphocytes were harvested by a pipette and Ficoll Paque was washed away with PBS. This cell fraction was used for the identification of the osteoclast precursor.

Preparation of Bone Marrow Cells, Spleen Cells, Hepatocytes, and Peritoneal Macrophages

Bone marrow cells were prepared by dissecting tibiae and femurs out of decapitated 2-week-old chickens and cutting the bones longitudinally into two pieces in DMEM-5% FCS. Bone marrow was carefully removed, suspended with a pipette, and filtered through a 50-μm Nybolt nylon filter. This cell suspension was used for the staining.

Chicken spleen cells were obtained by cutting little slashes on the spleen and by gently rubbing it with the rubber tip of a syringe piston. The large fragments of the cell suspension were

allowed to settle for 10 minutes. The supernatant was removed and used for the staining.

Chicken hepatocytes were prepared by washing the liver with PBS and by cutting it in small pieces. Liver tissue was digested with 0.05% collagenase (type IA, 750 U/ml, Sigma Chemical Co., St. Louis, MO, USA) in warm water (37°C) DMEM-5% FCS by stirring the tube for 30 minutes at room temperature (20 ml/liver). After digestion large fragments were allowed to settle for 10 min. The supernatant was filtered through a 50-μm Nybolt nylon filter and centrifuged for 10 minutes at 200 × *g*. The cell pellet was washed once with 50 ml DMEM-5% FCS. This cell suspension was used for the staining.

Peritoneal macrophages were isolated by intraperitoneal injection of 5 ml PBS in decapitated chickens. After gentle kneading of the abdomen, the PBS, containing resident macrophages, was withdrawn with a pipette. This cell suspension was used for the staining. The cells were stained as described above in Screening of Monoclonal Antibodies by Immunofluorescence Technique.

Acid Phosphatase Staining

Tartrate-resistant acid phosphatase staining was performed as described previously [32]. Different cell patches were also stained with routine hematoxylin and eosin staining in order to make the identification of cell types easier.

Results

The recovery of osteoclasts was 1×10^6 cells on average from three chickens weighing about 500 g each. Collagenase digestion was found to markedly improve the yield of intact osteoclasts when compared to mechanical dispersion. Also, the addition of FCS into the isolation medium improved the viability of the osteoclasts obtained when their capacity of attaching to cell culture dishes was tested. The purity of the osteoclast fraction was estimated to be about 70% by counting nuclei from smear preparations.

A total of 376 hybridoma colonies originating from one fusion were picked up from methylcellulose dishes, and 79 of these colonies (21%) grew well in multiwell dishes and tissue culture flasks. Primary screening of clones was done from the culture medium using the immunofluorescence screening method as described in Materials and Methods. The cell preparation used for screening contained all bone marrow elements, blood cells, and both types of bone cells, osteoblasts and osteoclasts. Fifteen of 79 clones detected osteoclasts. Several clones detected other cell types, for example, red blood cells but these clones were not studied further. Those 15 clones recognizing osteoclasts were expanded and some of them were injected into mice for the production of ascitic fluid.

In further screening, 13 of the 15 primary monoclonal antibodies were found to detect principally osteoclasts and a small population of so far unidentified mononuclear cells from bone marrow– and bone-derived cells. Mabs recognized chicken osteoclasts with different staining patterns, indicating that their specificities differ from each other. Mab K27 (Fig. 1) and mabs K34, K35, K38, and K70 (Fig. 2) stained the whole osteoclast strongly and evenly. Mabs K5, K24, K36, K47, and K52 seemed to stain only some part of the osteoclast (Figs. 1–3). In addition, some of these mabs labeled a few large mononuclear cells derived from bone. In the mononuclear cells the staining intensity was marked and no clear-cut polarization in the fluoresence was observed. K2 stained only multinuclear osteoclasts. Interesting, K5 stained some osteoclasts, but not all (Fig. 1c, d). Besides osteoclasts the positive immunofluorescence reaction with K5 was seen in thrombocytes. Mabs K41 and K51 stained osteoclasts weakly and uniformly, but some mononuclear cells in bone and peripheral blood were stained strongly and evenly (Fig. 4).

Staining specificity was confirmed by using NS-1 culture medium instead of primary antibodies. All monoclonal antibodies were immunoglobulin G_1 (IgG_1) type determined by the Ouchterlony immunodiffusion technique. Our preliminary studies suggest that there was no cross-reaction with rat osteoclasts.

The specificity of the monoclonal antibodies to osteoclasts were further studied by letting the monoclonal antibodies react with chicken cells of different origin (Table 1). The most specific mabs for osteoclasts turned out to be K2, K24, K27, K34, K36, and K38. These mabs have been found to recognize only osteoclasts although several other cells have been tested. A monoclonal antibody K41 recognized, in addition to osteoclasts, a few mononuclear cells in the peripheral blood of egg laying hens, but not in bone marrow or other tissues. K47 and K52 also recognized some mononuclear cells in the bone marrow. The rest of the monoclonal antibodies recognized, in addition to osteoclasts, some or many different other types of cells derived from other tissues. K35 labeled most of the cells in all tissue studied. K70 also stained various cells in different tissue. In the liver preparation most of the cells were strongly positive. These positive cells were recognized as hepatocytes based on their large size, typical nuclear shape, and location as well as abundant eosinophilic cytoplasm.

When the mononuclear bone cell fraction was obtained from 40–50% Percoll density layer and stained with monoclonal antibodies, three mabs—K38, K52, and K70—recognized almost the same amount of cells, varying between 2.6 and 3.4% (Ta-

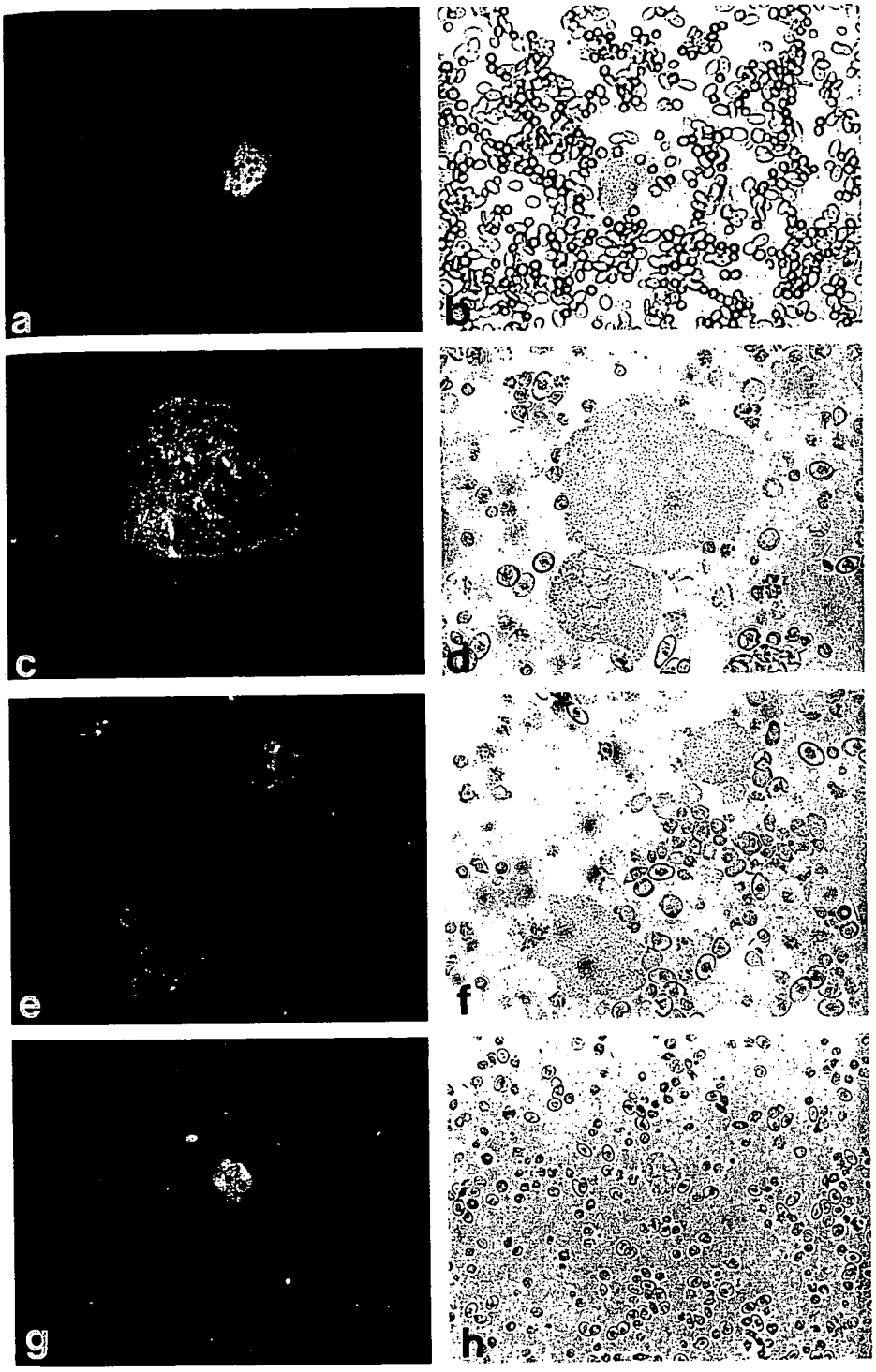

Fig. 1. Chicken bone and bone marrow cells recognized by monoclonal antibodies and stained with FITC-conjugated antimouse immunoglobulins. **(a), (c), (e), and (g)** are stained with monoclonal antibodies K2, K5, K24, and K27, respectively, and **(b), (d), (f), and (h)** are phase contrast pictures of the same area. Only osteoclasts are stained and their staining patterns are different from each other, suggesting the detection of different antigen by each clone (×170 [a, b]; ×340 [c–f]; ×264 [g, h]).

ble 2). The same cell fraction was also stained for TRAP in order to see if it matched with mab-positive cells. The amount of TRAP-positive cells was, however, smaller (1.9%) than in mab-positive cells. In the mononuclear cells fraction enriched in Ficoll Paque centrifugation there were mab-positive cells—0.8% on average (Table 2).

Five monoclonal antibodies—K41, K47, K51, K52, and K70—recognized few cells in the peripheral blood of egg laying hens. From these five mabs

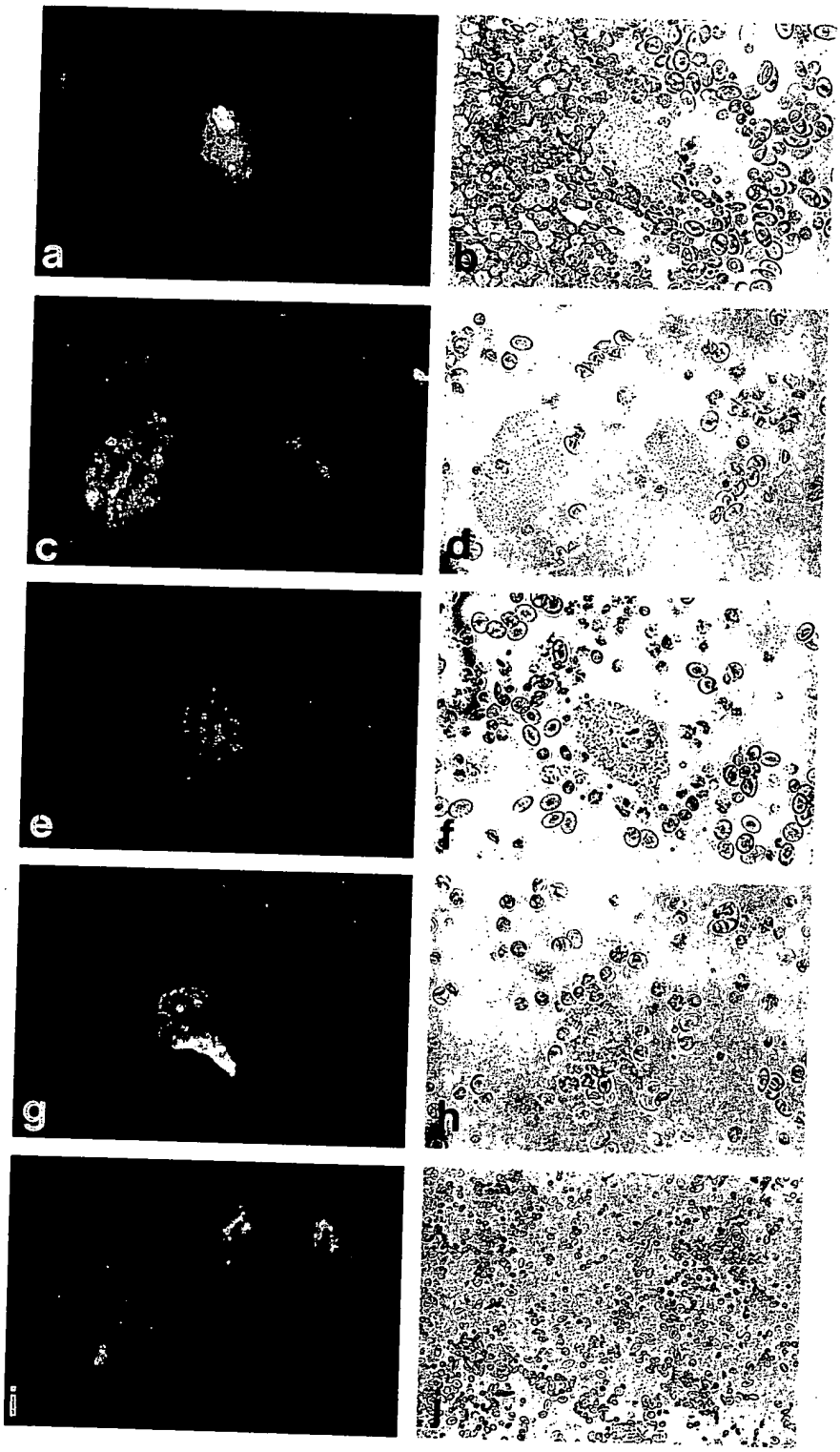

Fig. 2. These monoclonal antibodies show a strong diffuse membrane reaction only in osteoclasts. (a), (c), (e), (g), and (i) are stained with monoclonal antibodies K34, K35, K38, K36, and K70, respectively, and (b), (d), (f), (h), and (j) are phase contrast pictures of the same area (×272 [a–h], ×136 [i–j]).

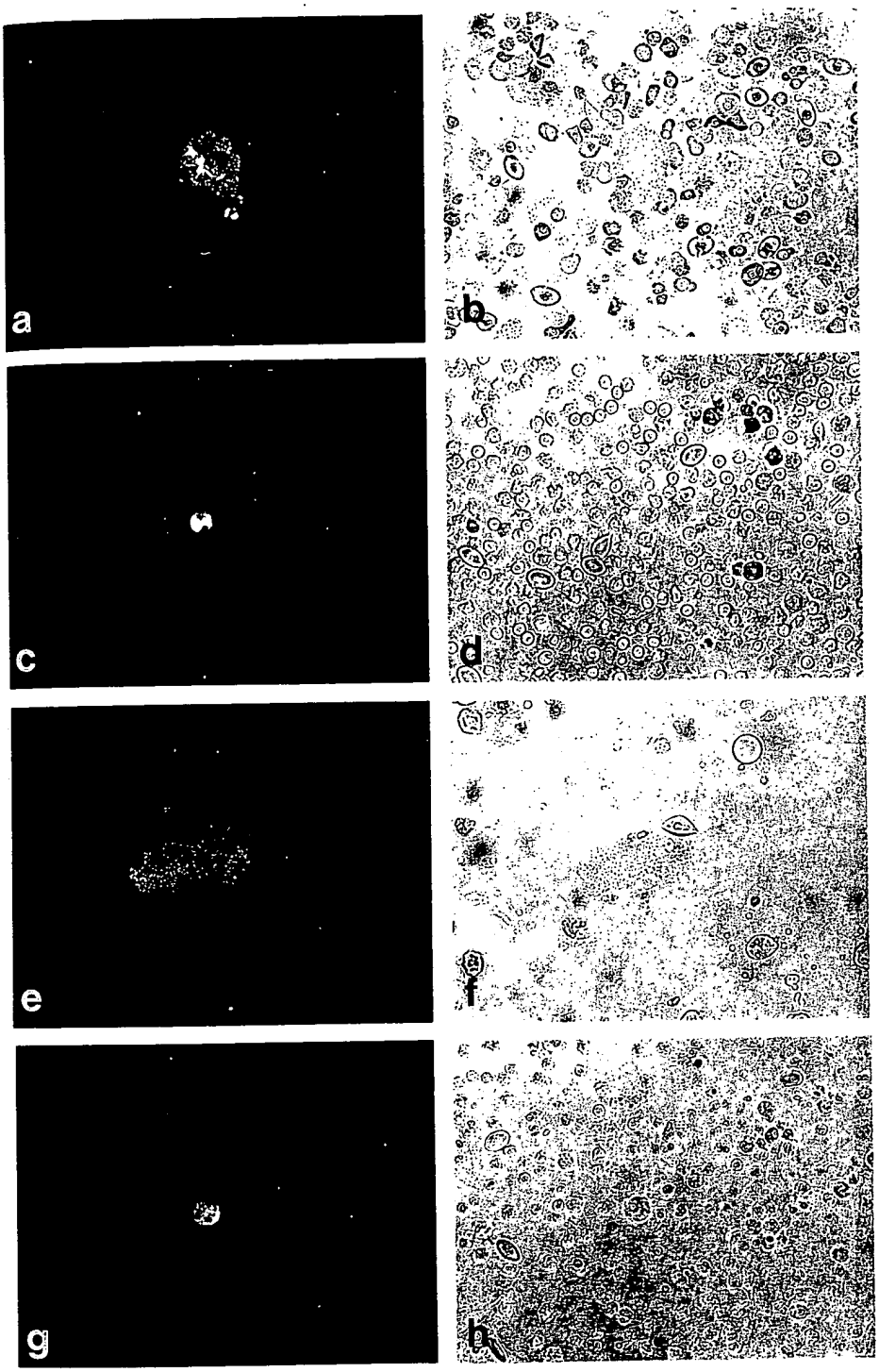

Fig. 3. Monoclonal antibodies K47 (a–d) and K52 (e–h) also recognize, in addition to osteoclasts (a, b and e, f), some large mononuclear cells in the peripheral blood of egg laying hens (c, d, and g, h) (×340).

the most specific for osteoclasts were K41, K47, and K52. They did not stain cells derived from other tissues, like spleen, liver, and peritoneal cavity. The quantity of positive cells in the white blood cell fraction was about 0.1%. These cells were also TRAP-negative (data not shown).

Discussion

By using a panel of chicken osteoclast-recognizing monoclonal antibodies we have identified mononuclear cells from chicken medullary bone that could be separated from osteoclasts in Percoll density

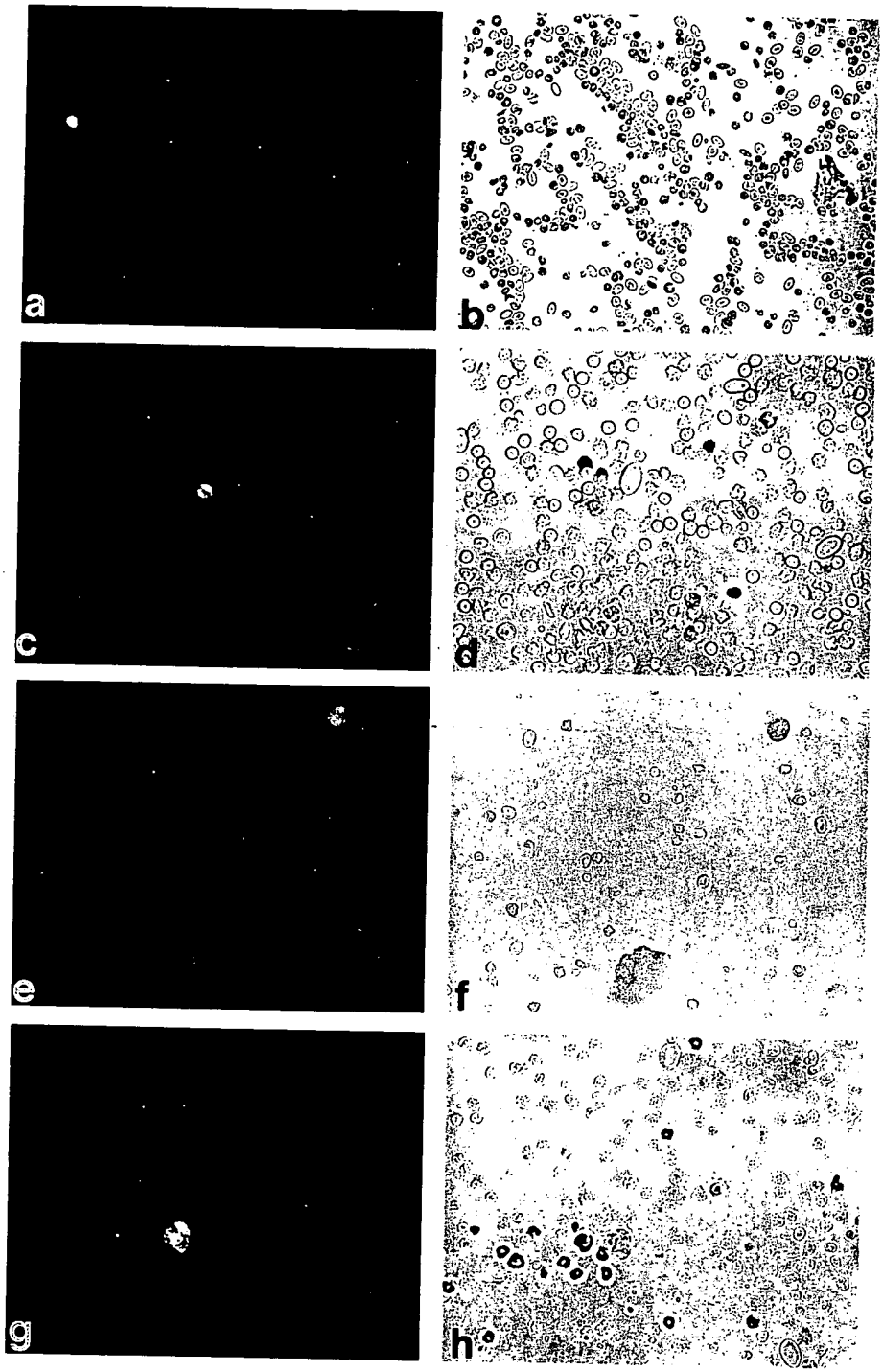

Fig. 4. Monoclonal antibodies K41 (**a–d**) and K51 (**e–h**) also recognize, in addition to osteoclasts and some bone marrow– or bone-derived mononuclear cells (**a, b** and **e, f**), some mononuclear cells in the peripheral blood of egg laying hens (**c, d** and **g, h**). Mononuclear cells show stronger staining than osteoclasts (×170 [**a, b**]; ×264 [**e, f**]; ×340 [**c, d, g, h**]).

centrifugation. These mab-positive cells represent 3% of obtained cell fraction. These same cells have probably also been identified earlier in feline bone marrow [5] and in the medullary bone of quails [33]. Those cells were accumulated on the 40–50% Percoll density layer [33] and were enriched there up to 30-fold as compared to unfractionated cells [5]. We found that in the mononuclear cell fraction of the bone enriched in Percoll density centrifugation the amount of TRAP-positive cells was less than mab-positive mononuclear cells. Double-staining with a monoclonal antibody and for TRAP

Table 1. Reactivity of antiosteoclast monoclonal antibodies with chicken cells of different origin

Mab	Osteoclast	Bone marrow[a]	Peripheral blood[a]	Spleen cells	Hepatocytes	Peritoneal macrophages
K2	++	−	−	−	−	−
K5	++	+	+[b]	+[b]	−	−
K24	+	−	−	−	−	−
K27	++	−	−	−	±	−
K34	++	−	−	−	−	−
K35	++	+	−	+	+	+
K36	++	−	−	−	−	−
K38	++	+	−	−	−	−
K41	+	−	++	−	−	−
K47	+	+	++	−	±	−
K51	+	+	++	±	±	+
K52	+	+	++	−	±	−
K70	++	+	+	−	++	+

++ Strong reaction, +weak reaction, ±no reaction or very weak reaction, −no reaction

[a] Few so-far-unidentified mononuclear cells

[b] Positive reaction only in thrombocytes

showed that TRAP-positive cells were, in almost every case, mab-positive but that the contrary was not true (data not shown).

We have used the indirect immunofluorescent method for the primary screening of the mabs as well as for the studies of the tissue distribution of recognized antigens. The advantages of this method is a low background and its simplicity. However, it is possible that we have missed some cells with lower levels of stained antigens that might have been detectable using more sensitive methods like biotin-avidin–based immunostaining.

Recently it has been reported that the expression of the osteoclast-specific antigen was observed after a 6-day culture period in the cultures of monocytes enriched from the peripheral blood [34]. Freshly isolated peritoneal macrophages and adherent cells from bone marrow cultures did not react with that osteoclast-specific antibody [33]. Three monoclonal antibodies—K41, K47, and K52—that recognized few mononuclear cells in the peripheral blood of egg laying hens did not stain cells derived from other tissues, like spleen, liver, and peritoneal cavity. When we used the white blood cell fraction of young chickens, the same monoclonal antibodies stained only a very few mononuclear cells. Possibly during the egg laying when the recruitment of osteoclast precursor cells and the resorption activity is high, more osteoclast precursors are also present in the peripheral blood. Now these cells need to be separated from other cells and to be studied to determine whether they form osteoclasts under the appropriate stimuli.

Other researchers have also developed monoclonal antibodies against chicken osteoclasts which recognized three polypeptides (96, 91, and 45 kD) on Western blot. They also demonstrated that osteoclast antigens were present on giant cells and cultured monocytes [21]. Other osteoclast-recognizing mabs described so far were produced against human osteoclastoma cells [35] and quail osteoclasts [33]. The majority, 7 of 11 osteoclast-recognizing mabs, did not cross-react with the mononuclear phagocytes in a wide range of tissue, suggesting the difference between these two cell types [35]. Of five monoclonal antibodies against quail osteoclast, two recognized surface antigens present on osteoclasts, monocytes, granulocytes, and endothelial cells [33]. The three other monoclonal antibodies recognized osteoclasts in bone and other cells in different tissues: bile capillaries of the liver, unidentified parts of the nephron, Kupffer cells of the liver, and tissue macrophages of the small intestine. On the basis of these results the authors concluded that there was a common origin for osteoclasts and tissue macrophages [34].

Blood-borne precursor cells of the osteoclasts have been suggested by using chick-quail chimaeras [16] and parabiotic union of experimental animals [18]. These studies strongly suggested that the osteoclast precursor cells were present in the peripheral blood and probably even before the onset of osteogenesis [17].

Since the osteoclast is a phagocyte it has been presumed that the osteoclast precursor is a mononuclear phagocyte. First, monocytes and macrophages are attracted to bone [24, 25]. Second, it has been demonstrated that isolated blood monocytes have been able to fuse with isolated osteoclasts in *in vitro* cultures [26, 27]. In addition, *in vivo,* leuko-

Table 2. Identification of mononuclear mab-positive cells in chicken bone and bone marrow cell fractions

	Bone				Bone marrow			
Mab	n	Total amount of counted cells	Mab-positive cells	%	n	Total amount of counted cells	Mab-positive cells	%
K38	4	659 ± 173	19 ± 2	2.6 ± 1.0	3	642 ± 265	2 ± 0.3	0.7 ± 0.6
K52	3	280 ± 20	9 ± 1	3.1 ± 0.2	4	354 ± 42	2 ± 0.3	0.7 ± 0.2
K70	5	425 ± 35	16 ± 3	3.4 ± 0.4	5	238 ± 55	2 ± 0.8	1.1 ± 0.3

Values are means ± SEM, n = number of experiments

cytes, probably monocytes, from the female animal have been reported to fuse with male osteoclasts detected by the transfer of Barr bodies, suggesting a common origin of monocytes and osteoclasts [28]. Third, by using a marker enzyme, nonspecific esterase for mononuclear phagocytes it has been shown that mononuclear osteoclast precursors are members of the monocyte-macrophage lineage [19]. Fourth, both osteoclasts and monocytes have similar patterns of distribution of cytoskeletal structures and they both adhere to fibronectin-free areas [23]. However, there are also studies where any common markers, characteristic of mononuclear phagocytes, macrophages, granulocytes, inflammatory polykaryons, or leucocytes were not been able to find on the surface of osteoclasts [35–38]. Our results are in agreement with these studies and suggest that in the peripheral blood there is a cell population different from monocytes and that these cells may be preosteoclasts.

Osteoclasts contain TRAP [32]. TRAP-positive cells are also found among mononuclear cells in bone marrow and periosteum [39, 40]. TRAP-positive osteoclast precursor-like cells have been reported to have the characteristics of both mononuclear phagocytes and osteoclasts. Hence, it has been proposed that osteoclast precursor-like cells are in the relatively late stages of osteoclast-specific cell lineage diverged from the mononuclear phagocyte lineage [41]. It has also been suggested that osteoclast precursors are differentiated from a pool of mononuclear nonspecific esterase-positive cells. They increase in number locally, acquire TRAP-positive structures, and become later positive for TRAP [19]. We found that the mononuclear cells in the peripheral blood of egg laying hens recognized by the monoclonal antibodies K41, K47, K51, and K52 were not TRAP-positive. On the other hand, most of the mab-positive mononuclear cells in the enriched bone cell fraction were TRAP-positive. These data suggest that our mabs recognized antigens that were expressed earlier than TRAP in the maturation of osteoclast precursor cells.

Ostoeclasts, but not other bone cells, are rich in carbonic anhydrase II [42–44]. Lectins have been widely used for the identification of different cell types. Multinucleated rat and chicken osteoclasts are positive for peanut agglutinin (PNA)-lectin [45, 46]. PNA also recognizes some mononuclear cells on the endosteal surface of the rat calvaria, some bone marrow cells [45], and monocytes from the peripheral blood [45, 47]. Other characteristics of osteoclast precursors are that they are phagocytic and not initially adherent to plastic [5].

So far we do not have any detailed information about the character of the antigens that these 13 monoclonal antibodies recognize. According to their staining patterns they recognize different epitopes on the surface of the osteoclast. There was some polarization in the staining patterns, suggesting that the mabs K5, K24, K36, K47, and K52 possibly recognized some specialized membrane area of the osteoclast. Two of 13 osteoclast-recognizing mabs also labeled epithelial cells (hepatocytes), indicating the presence of some common epitopes in these otherwise different cells.

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